[redacted]

February 18, 1970

[redacted]

COPY 2 OF 6 COPIES

Dear [redacted]

Enclosed are five copies of the third quarterly progress report for the present contract, [redacted] and covers the interval from October 1, 1969 to December 31, 1969. The report includes a statement of expenditure of funds and completion of the assigned task.

Please let us know if there are any questions or comments as to its contents.

Sincerely yours,

[redacted]

[redacted]

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[redacted]

[redacted]

COPY 2 OF 6 cc

THIRD QUARTERLY REPORT

ABSTRACT

Areas of activity during the third quarter are listed in this paragraph and elaborated below. Progress continued on an accelerated level on the basic toxicity and behavioral screening programs, and additionally included cardiovascular pharmacodynamic testing of a group of compounds and isolation and testing of extracts of several oriental plant products. A total of 53 compounds were received for screening during this time interval. In addition, a natural products chemist began work on preparation of extracts of oriental plants reportedly of medicinal value according to Chinese folklore. Several of these plant extracts have been tested for biological activity and toxicity in mice; work has proceeded to the point of separation of several active fractions. The visual discrimination apparatus has been received from [redacted] and assembled. To date 61 per cent of the funds have been expended and 61 per cent of the task completed.

[redacted]

[redacted]

PRIMARY SCREENING

During the current reporting period, 36 compounds were tested in mice by the acute toxicity screen and locomotor activity tests. Practically all of these compounds showed a suitable minimum safety ratio between the LD_{50} and MED_{50} to warrant further testing. Five compounds showed outstanding safety ratios (1000 or greater). These were [redacted] and [redacted]. In addition, 10 compounds showed ratios between 100 and 1000. However, all compounds were tested in cats at a dose (mg/kg) 1/10 the mouse LD_{50} level, for the effect on physical, neurological, and behavioral status. Ten of these compounds showed significant activity.

SECONDARY AND ADVANCED BEHAVIORAL SCREENING

Thirty-four compounds were evaluated in hooded rats by the motivation and sequential response behavioral tests. The dosage (mg/kg) administered was 1/20 the mouse LD_{50}, or lower if overt toxic signs were evident in preliminary test rats. In both the motivation and the SRBA tests a number of compounds showed some psychopharmacologic activity, as evidenced by an increase or decrease in start or run speeds or a decrease in the number of rewards and an increase in the percentage of errors. For further evaluation, test compounds were administered to monkeys, and the physical, neurological, and behavioral effects, including changes in shuttle order behavior in the shuttle box test, were determined. This is further described in detail in a separate section below. Sixteen compounds were studied in monkeys.

PHARMACODYNAMIC SCREENING

Pharmacodynamic screening was carried out on 18 compounds in anesthetized cats. The protocol for the procedure and the results obtained are described below.

Pharmacodynamic Screening in Anesthetized Cats

Compounds were screened for pharmacodynamic activity in cats anesthetized with α-chloralose (80 mg/kg, i.p.). Arterial blood pressure, heart rate, respiratory movements, and EKG's were recorded on an E and M Physiograph. Effects on responses to the administration [redacted] and to vagal stimulation and to carotid artery occlusion were measured. Challenging drugs were usually administered at five-minute intervals or after arterial pressure had returned to the control level. All injections were made via polyethylene cannula inserted into the femoral vein.

Each compound was usually tested in one animal. The initial dose was either 0.3 or 1.0 mg/kg. Subsequent doses were increased or decreased by one-half of a logarithmic interval, depending on whether a response was elicited.

The results of these experiments are summarized in the Appendix. Intrinsic activity was observed with 15 of the 18 compounds tested. One compound [redacted] caused an increase in blood pressure and 12 compounds caused decreased blood

pressure. The duration of each of the blood pressure changes was less than five minutes. EKG voltage increases were observed after administration of [redacted] None of the compounds affected the responses to any of the challenging drugs or procedures.

BEHAVIORAL TESTING OF SQUIRREL MONKEYS

Certain personnel additions, procedural implementations, and equipment modifications were instituted to facilitate the operation of the squirrel monkey testing program.

Personnel Additions - A behavioral scientist was added to the staff and assigned to the squirrel monkey testing procedure.

Equipment Modifications - Basic additions to increase the efficiency of the testing procedures and to insure the validity of results obtained are listed below:

a. The testing area containing the shuttle box was isolated from the data-recording area and the investigator by a booth made of sheets of opaque black plastic. Both the investigator's and testing areas were then similarly separated from the general room environment. Thus, the room lights could remain lit during testing procedures so that the untreated monkeys would be exposed to a normal light-dark cycle.

b. A one-way mirror was placed between the area of the investigator and that of the test subject, so that the monkeys performed their tasks while being visually isolated from the influence of the investigator or general room movement, but fully observed by the investigator.

c. General fluorescent lighting was placed in the area of the shuttle apparatus to provide an overall illuminance so the monkeys could readily see the visual cues and the investigator could easily determine the monkeys' identification and behavior patterns.

d. A mechanical door closing system for the shuttle boxes was added so that the entire testing operation, after the monkey was placed in the shuttle box, remained visually isolated from the monkeys. This further reduced intra-test disturbances and variability.

The above modifications were designed to isolate as much as possible the test animal from any distracting situations, both visual and auditory. This is essential for the propagation of motivational data and response consistency.

e. A styrofoam weighing cage and a plexiglass injection tube were constructed to reduce mechanical injuries due to handling, contact, and infection from abrasion.

Procedural Changes - Certain changes were made to maximize the data presently obtained and to provide for an increase in data in the future.

a. Scheduled training sessions were initiated. These will continue until the projected full complement of trained monkey groups is attained. At present, one shuttle group of three monkeys trained to both a positive and negative

reinforcement schedule and one group of three trained to a negative regime are available for group shuttle testing. In addition, seven males, which can be trained on a positive reinforcement schedule to replace any established monkeys or which can be used for the visual discrimination testing program, are in the colony.

b. Seven female squirrel monkeys were purchased and conditioned to the shuttle box apparatus. These are used daily to determine a dosage level of test compound (mg/kg) which produces minimal behavioral effects and can thus be applied in a shuttle operation. These monkeys are important in the detection of behavioral effects, observed in the shuttle apparatus or in the home cage, caused by varying drug dosage levels. The procedure on individuals includes the observation of many behavior patterns and motivational states and gives much information about a specific drug effect which may be present.

(1) Observational Procedure and Criteria. The individual is observed and subjected to the stimuli presentations every five minutes in the shuttle box for a 30 minute control period prior to dosing. The first dose is injected, and the animal is placed back in the shuttle box and observed for one hour. Subsequent increasing dose levels proceed hourly until the maximum determined dose is reached (cumulatively). The individual is then observed in its home cage after four hours and after 24 hours. The behavioral activities being cataloged during this procedure are: (1) reactions to observation and handling by the investigator (flight, attack, or passiveness); (2) grasping ability (to bars and manipulanda); (3) feeding behavior (eating or not); (4) reaction to blinking light (fixation, orientation, alert response); (5) reaction to mild shock (immediate jerk, tremor, hop, etc.);

(6) reaction to sound of buzzer (alert, orientation, etc.); (7) shuttle response (ability to shuttle after light cue, door open, and shock if used); (i) level of self-manipulative behavior (grooming, self-inspection, etc.); (9) locomotor coordination, speed, ability (natural, under stimulation and during shuttle); (10) level of general activity; (11) amount of sleep; (12) behavior during crouching (alertness, head orientation responses, eye fixation).

(2) Injection Procedure. All injections of test compounds are given intravenously via a caudal vein. The total dose level is determined by extrapolation using as a base the lower limit of the 95 per cent confidence limits of the LD_{50} for mice. The dose to monkeys was calculated using the conversion formula of Paget and Barnes[1] which is based on surface area; also by reference to Dr. Elton Homan's equivalent surface area dosage conversion factor card as reported by Freireich[2]. The maximum dose level (mg/kg) administered to a squirrel monkey as extrapolated from the dose to a mouse to yield an equivalent dose based on surface area is determined by multiplying the mouse dose (mg/kg) by a factor (0.45). Aliquots of this total dose are then given in one hour intervals until effects are observed or the extrapolated dose level is reached.

REFERENCES

1. Paget, G.E., and Barnes, J.M., 1964. Chap. 6, Toxicity Tests in Evaluation of Drug Activities, Pharmacometrics, P.R. Laurence and A.L. Bacharach (eds.). Vol. 1, Academic Press, pp. 135-167.

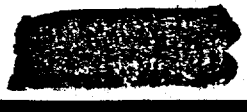
2. Freireich, E.G., et al., 1966. Quantitative Comparison of Toxicity of Anti-cancer Agents in Mouse, Rat, Dog, Monkey, and Man. Cancer Chemotherapy Reports, 50(4), 219-244.

[redacted]

NATURAL PRODUCTS

1. Chinese Toxic Compounds: The names of 47 intact plants, roots, tubers, and flowers were selected from a medieval Chinese medical and pharmaceutical reference (Li Shih-Chen, 1551). Botanical names of these plants are as follows:

1. Rheum officinale
2. Phytolacca esculenta
3. Peucedanum Japonicum Thunb.
4. Potentille cryptotaemiae maxim
5. Euphorbia adenochlora Morr et Dene
6. Euphorbia sielaldiana morren et Decaisne
7. Euphorbia heliscopia L.
8. Galarhoeus siebaldianus Hara.
9. Semen Euphordiae Lathyris
10. Hyoscyamus agrestis Kitail
11. Unjitsu (unknown in Western terminology)
12. Richinus communis L.
13. Orixa japonica Thunb. (Dichroa febrifuga)
14. Andropogon Sorghum Brat. var. vulgaris Hack
15. Veratrum nigrum L.
16. Leucothoe Grayana Maxim
17. Aconitum Fischri Reich
18. Tenyuh. (unknown in Western terminology but similar to Aconitum Fischori)
19. Aconitum Chinese Sieb. (daughter root)
20. Aconitum Chinese Sieb (small root)
21. Aconitum Sinesis
22. Jatropha Janipha
23. Rhizoma Arisaematis
24. Arisaema ringeus Schott var. Sieboldi Engl.
25. Hydrosme Rivieri Engl.
26. Pinellia Tuberifera
27. Polygonum bistorta L.
28. Podophyllum versipella Hee.
29. Pardanthus sinensis
30. Iris tectorum Maxim
31. Hosta Sieboldiana Engl.
32. Kaempferris Galanga
33. Zachoso (not in Western terminology)
34. Stramonium Datura Alba
35. Rhododendron Sinense Sa.
36. Daphne Genkwa S. et. Z.



37. Wikstroemia Japonica Miq.
38. Baddlea Japonica Hemsl.
39. Illicium anisatum L.
40. Shimmia Japonica Thunb.
41. Ranunculus sceleratus L.
42. Ranunculus acer. L. var. Japonicus Maxim.
43. Aconitum Lycoctomum L.
44. Urtica Thunbergiana S. et Z.
45. Gleditschia glauca HK
46. Alocasia Macrorhiza Schott
47. Rhus Toxicodendron L. var. Radicans Miq.

Plants numbered 4, 13, 17, 22, 28, 29, 30, 31, 34, 35, 37, 38, 42, 43, and 44 have been checked by the Natural Product Section of the National Cancer Institute and were negative in anti-tumor tests. However, several fruit-bearing Euphorbia species showed activity against Sarcoma 180, Walker 256, and Lewis lung carcinoma. Several of the active principles are in the process of fractionation, but in only one case was the active agent isolated and this was reported to be tannin. Dichroa fibrifuga root was active against KB cell culture. This plant is being fractionated, but the active agent has not yet been isolated. The rhizome of one species of Arisaema was active aginst Lewis lung carcinoma, but the active compound has not been identified.

Twenty-four species selected from the above list of materials were ordered from a Chinese pharmaceutical distributor, [redacted]. Because the [redacted] is encountering some delay in acquisition, these 24 species also have been ordered from other sources in [redacted]

In addition to the plant materials described above, a Chinese pharmaceutical and extracts of black tea, green tea, and aronia fruit were tested for toxicity in mice.

[redacted]

Some [redacted] a Chinese pharmaceutical, was obtained from [redacted] The chemical composition of the material is unknown. Ten milliliters of distilled water was thoroughly mixed with 1.125 grams of this compound, and the material was injected in mice. No effect was observed. In the appended computer printout tables, this material is reported as No. 300000.

The tea extracts showed some activity in mice; however, it is likely that the effects of caffeine masked any pharmacologic action by other ingredients. The tea extracts are designated in the appended computer printout as follows:

Extracts of green tea: Nos. 110000, 120000, and 130000
Extracts of black tea: Nos. 140000 and 150000.

Fruits of the aronia plant (Malus Halliana Kochne) were harvested during the winter from trees located [redacted] after all the leaves had fallen and only brownish red fruits remained. The fruits were immediately frozen and stored at -15° with dry ice in a styrofoam cabinet. Frozen fruits (32.51 grams) were blended first and then homogenated with an electric blender for 10 minutes and extracted twice with 100 milliliters of distilled water by stirring under nitrogen for two hours at room temperature. The filtrates were combined and concentrated to dryness using a freeze-drying procedure. The residue of the filtrate was re-extracted twice with 100 per cent acetone (100 milliliters). The acetone extracts were combined and evaporated to dryness under a vacuum.

[redacted]

The residues from the water extract fraction and the acetone extract fraction were each made up to a volume of 32 milliliters with distilled water. Each milliliter contained the extractable material equivalent to one gram of freshly weighed fruit. Aliquots (40 ml/kg, 16 ml/kg, and 5 ml/kg) of these solutions were injected intravenously in Dublin (DUB/ICR strain) albino mice via a tail vein. The effects of the injections are presented in the computer assembled tables (fraction numbers 220000 - acetone, 210000 - water) and show that an active compound was soluble in acetone. The mice died during, or immediately after intravenous injection of a dose of 16 ml/kg of the acetone fraction.

The previous experiments showed that at least one toxic compound was distributed in the acetone fraction, so the compound(s) was (were) further separated and purified with a series of organic solvents since the appearance of the acetone soluble material(s) in a particular solvent fraction would provide information concerning the polarity and chemical properties of the compound(s).

Another quantity (61.23 grams) of fruits was blended with an electric blender and extracted twice with 200-milliliter aliquots of n-hexane (dielectric constant 1.9), chloroform (4.8), diethyl ether (4.3), acetone (20.7), ethyl alcohol (24.3), and distilled water (78.5). The extractions were carried out at room temperature. Insoluble material was filtered from the extraction fractions using Whatman No. 1 paper.

Fraction	Weight g	Per Cent of Fresh Weight	Biological Activity
n-hexane	0.322	0.52	
chloroform	0.521	0.85	++
ethyl ether	1.157	1.88	
acetone-1	0.456	0.74	
acetone-2	5.668	9.80	+++
ethyl alcohol	2.0917	3.41	
distilled water	1.331	2.17	+

As shown above, at least two active compounds were extracted from the aronia fruits. The designations "acetone-1" and "acetone-2" indicate different steps of fractionation; the residue following the diethyl ether extraction was extracted twice with 200 milliliters of 100 per cent actone. The acetone extracts were filtered and the filtrate was evaporated to dryness. The sap of the fruit is miscible in acetone, and therefore it was possible to extract a polar compound in the acetone. The residue was taken once completely to dryness in a desiccator and then re-extracted with acetone (acetone-1). The residue of the acetone extract was freely soluble in water (acetone 2).

One-tenth aliquots of each of the residues from each extract were dissolved in 6.2 milliliters of distilled water from which portions (40 ml/kg, 16 ml/kg, or 5 ml/kg) were injected in mice. Nonpolar solvent extract fractions were suspended in 0.5 per cent methylcellulose and injected in the same volume as the polar fractions. The nonpolar fractions were n-hexane, chloroform, diethyl ether, and acetone-1. The water fraction contained an active compound which resembles the active compound in the acetone-2 fraction. The results of biological testing are shown in the appended computer prinout tables. The acetone-2 extract is No. 214200 and the water extract is No. 21600.

At least two biologically active compounds were separated from the aronia fruits by the described procedures. One is nonpolar and the other is a polar compound. No difference in biological activity was observed after the pH of the extracts was adjusted

[redacted]

with sodium hydroxide to 6.8 from 2.5 - 3.0 in the acetone, ethyl alcohol, or water fractions.

The active compound in the acetone-2 fraction was purified further. Four hundred sixty-nine milligrams of the acetone-2 fraction residue was dissolved in 0.5 milliliter of distilled water. The water solution was streaked onto six TLC plates, which were Brinkman precoated and had a 0.25 mm thickness of Silica Gel-G containing $CaSO_4$ as a binder. The TLC plates were developed at room temperature with an ethanol (96 per cent):water:ammonia solution (23 per cent), 100:12:16 v/v, solvent system (Braum and Genneu, 1962, J. Chromatg. 7, 56). Chromatographic separation continued for eight hours.

The plate was divided into 10 parts on the basis of Rf values. Each part was scraped off with a spatula and suspended in distilled water. The water extracts were lyophilized and the residue was dissolved in 3.1 milliliters (two times concentrated solution of 6.2 milliliters*) from which portions were used in the bioassay.

The Separation of Active Compound of Acetone-2 Fraction Using Thin-Layer Chromatography

Fraction No.[1]	Weight of Material With Binder	Weight of Binder	Biological Activity
1	167.1	29.8	±
2	132.2	29.8	-
3	126.0	29.8	-
4	79.7	29.8	++
5	47.2	29.8	+
6	45.8	29.8	-
7	45.4	29.8	-
8	48.8	29.8	-
9	47.6	29.8	-
10	45.0	29.8	-
TOTAL	784.8	298.0	

[1] Correspond to Rf values. Recovery of experiment procedure was (784.8 - 298) = 486.8 486.8/569 x 100 = 85.5%

* Original weight of the material was 569 mg from 6.2 g of fresh weight

The active compounds located in the acetone-2 fraction have not been completely purified yet, so they have not been identified. This will be done in the future, as will the purification of the active compound which is soluble in chloroform.

The aronia extracts which were active, showed an interesting activity, suggestive of that produced by several types of compounds, such as stimulants, morphine-like drugs, or hallucinatory agents.

GOALS FOR NEXT PERIOD

Work will continue on all aspects of the behavioral screening as described in this report. Additional compounds will be obtained for testing. Training of monkeys in the visual task discrimination will be initiated. Natural product isolation will be continued and extracts tested. Pharmacodynamic screening in anesthetized cats will also be continued.

Submitted: February 13, 1970

[redacted]